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Metabolite Profiling and Anti-Inflammatory Potential of East Kolaka Forest Honey: Targeting Protein Denaturation through GC-MS/MS Characterization

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ABSTRACT

Forest honey is a natural product produced by wild bees that gathers nectar from various types of flowers, creating a unique chemical composition influenced by the biodiversity of the surrounding environment. This honey contains bioactive compounds such as phenolics, flavonoids, terpenoids, and volatile substances that contribute to its pharmacological properties, particularly as an anti-inflammatory agent. This study aimed to identify secondary bioactive metabolites in forest honey from East Kolaka using Gas Chromatography-Mass Spectrometry (GC-MS) and to evaluate its anti-inflammatory activity through in vitro assays, including red blood cell (RBC) membrane stabilization, protein denaturation inhibition, and nitric oxide (NO) production inhibition. GC-MS analysis revealed the presence of various bioactive compounds such as phenolic acids, esters, and fatty acids, which are known to possess antioxidant, antimicrobial, and anti-inflammatory activities. The RBC membrane stabilization assay showed increased membrane stability as honey concentration increased, reaching 92.55% at 100 mg/L compared to 97.00% for sodium diclofenac. In the protein denaturation assay, East Kolaka forest honey exhibited strong anti-inflammatory activity with an IC_{50} of 2.97 ± 1.34 mg/L in the NO inhibition assay and moderate activity in the protein denaturation test IC_{50} = 12.70 ± 0.02 mg/L, comparable to vitamin C IC_{50} 1.07 ± 0.39 mg/L. Statistical tests showed significant differences ($p < 0.05$) in all tested parameters compared to positive controls. These results demonstrate that East Kolaka forest honey exhibits potent anti-inflammatory activity mediated by membrane stabilization, protein protection, and nitric oxide inhibition mechanisms.

Keywords: GC-MS, Honey, HRBC, Nitric Oxide, Protein Denaturation

INTRUDOCTION

Honey is a natural product produced by bees from the nectar of various flowers through enzymatic transformation. Its chemical composition is strongly influenced by nectar sources, bee species, and environmental conditions, resulting in a diverse metabolite profile (Mokosuli et al., n.d.; Palma-Morales et al., 2023). Forest honey produced by wild bees, such as *Apis dorsata*, generally contains a higher diversity of secondary metabolites compared to cultivated honey, which contributes to stronger pharmacological activities. The unique flora of East Kolaka imparts specific chemical characteristics to forest honey from this region, making it a potential natural product with unique bioactive properties (Palma-Morales et al., 2023).

East Kolaka Regency is recognized as one of the top three honey-producing regions in Southeast Sulawesi, supported by extensive forest ecosystems that serve as natural habitats for wild bees. Forest honey from this area is typically produced by *Apis dorsata*, a wild bee species known for generating honey with distinctive organoleptic characteristics and high pharmacological potential (BPS, 2024). However, despite its potential, scientific data on the chemical composition and pharmacological activity of East Kolaka forest honey remain limited.

The biological activity of honey is mainly attributed to bioactive compounds, including flavonoids, phenolic acids, terpenoids, fatty acids, and volatile compounds, which exhibit antioxidant, antimicrobial, and anti-inflammatory effects (Ndungu et al., 2024). Several major compounds reported to contribute to these activities include 2,4-

di-tert-butylphenol, palmitic acid (n-hexadecanoic acid), and methyl palmitate (Nair et al., 2020; Aparna et al., 2012). These compounds have been shown to inhibit inflammatory mediators such as cyclooxygenase (COX) and *nitric oxide* (NO), thereby reducing inflammation and oxidative stress in tissues(Alaerjani & Mohammed, 2024; Balazs et al., 2023). In addition to non-volatile compounds, volatile compounds in honey also contribute to its biological effects.

Previous studies have identified volatile compounds such as benzaldehyde, octanoic acid, and nonanal, as well as aldehydes, ketones, alcohols, terpenoids, and aromatic esters that enhance the therapeutic properties of honey (Sotiropoulou et al., 2021). However, characterization of volatile and semi-volatile compounds in forest honey from East Kolaka remains limited, highlighting the need for further investigation into secondary metabolites responsible for anti-inflammatory activity(Alghamdi et al., 2020; El Maimouni et al., 2024; Sotiropoulou et al., 2021).

Inflammation is the body's natural response to injury or tissue damage, characterized by swelling, heat, pain, and redness (Fristiohady et al., 2019; Fristiyohadi et al., 2019). This process aims to protect the body by repairing damaged tissues and combating irritants such as physical trauma, chemicals, or microbial infections (Moutawalli et al., 2024; Zubaydah et al., 2019). Inflammation is categorized into two types: acute inflammation, which is temporary, and chronic inflammation, which persists longer and is often associated with degenerative diseases like arthritis and diabetes (Hulea et al., 2022).

Long-term use of anti-inflammatory drugs carries risks of side effects, such as gastric ulcers and kidney disorders. Therefore, safer natural alternatives, like honey, become attractive options. Bioactive compounds in honey, particularly flavonoids, phenolics, terpenoids, and volatile compounds, have been shown to exert anti-inflammatory effects through multiple mechanisms inhibiting protein denaturation, stabilizing red blood cell (RBC) membranes, and suppressing nitric oxide production, all of which reduce inflammatory responses and oxidative stress (Bhavikatti et al., 2024; Suryani et al., 2023). Understanding the direct relationship between secondary metabolites and their anti-inflammatory mechanisms is essential for developing honey as a therapeutic agent (Alaerjani & Mohammed, 2024; Hariana & Fristiohady, 2025; Hulea et al., 2022).

In this context, Gas Chromatography–Mass Spectrometry (GC-MS/MS) is used as an analytical tool to identify volatile and non-volatile bioactive compounds in forest honey from East Kolaka. It is important to note that GC-MS provides chemical composition information only, while the biological activity of the compounds is evaluated experimentally through in vitro assays, including RBC membrane stabilization, protein denaturation inhibition, and nitric oxide suppression (Ndungu et al., 2024; Xiao et al., 2021).

Based on this background, the present study aims to identify the chemical compounds in East Kolaka forest honey using GC-MS/MS and evaluate its anti-inflammatory activity in vitro, providing a strong scientific basis for the development of East Kolaka forest honey as a natural therapeutic agent and supporting the sustainable utilization of local resources with high potential.

RESEARCH METHODS

Sample Preparation

Forest honey samples were collected from natural forest areas in East Kolaka Regency, Southeast Sulawesi, Indonesia geographical coordinates: 3°58′3.61″LS, 121°49′36.91″BT (−3.967669, 121.826919). The honey was identified as forest honey based on its physicochemical characteristics and confirmation by local apiarists. A 1-gram sample of this forest honey was subjected to sonication using an sonicator (Elmazonik®, India) with 10 mL of 70% ethanol (Onemed®, Indonesia) for 30 minutes at room temperature to ensure thorough mixing.

Following homogenization, the mixture was filtered through filter paper, transferred into a 100 mL volumetric flask, and diluted with ethanol up to the calibration mark, resulting in a sample concentration of 10.000 mg/L. The prepared sample was then stored at a temperature of 2–4°C until further analysis for its anti-inflammatory activity (Hulea et al., 2022).

Compound Analysis using Gas Chromatography-Mass Spectrometry (GC-MS)

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the honey sample was conducted following the procedure outlined by (Joshna, 2022). Initially, 1 gram of honey was dissolved in 2 mL of distilled water and homogenized using a shaker for 10 minutes. Subsequently, 10 mL of ethyl acetate and 10 grams of anhydrous sodium sulfate (Merck®, Germany) were added to the mixture to remove water content. The solution

underwent sonication for 10 minutes to enhance the extraction of bioactive compounds.

After sonication, the mixture was filtered through filter paper to eliminate solid residues. The resulting filtrate was then subjected to evaporation via sonication until all the ethyl acetate solvents had evaporated. The dried residue was reconstituted in 1 mL of n-hexane (Merck®, Germany) and transferred into a clean, tightly sealed GC vial for analysis. The GC-MS analysis was performed using an Agilent 8890 GC system coupled with a Xevo® TQ-GC mass spectrometer (UK). A DB-5MS column (30 m × 250 µm × 0.25 µm) was employed, with helium as the carrier gas at a flow rate of 1 mL/min.

The oven temperature program commenced at 110°C (held for 3.5 minutes), followed by a ramp of 10°C/min to 200°C (held for 1 minute) and a subsequent ramp of 5°C/min to a maximum of 280°C (held for 12 minutes), culminating in a total analysis time of 41.5 minutes. The inlet operated in splitless mode at 280°C with a 1 µL injection volume. Mass spectrometry detection was conducted in scan mode, with the MS source at 200°C and the GC-MS interface at 250°C, analyzing a mass range of 50–500 m/z using electron ionization (EI+) mode.

Anti-Inflammatory Activity Assay

1. Red Blood Cell Membrane Stabilization Assay

The anti-inflammatory activity of the honey sample was evaluated using a red blood cell (RBC) membrane stabilization assay, adapted from (Fristiohady et al., 2020) with minor modifications. Fresh blood samples were collected from healthy volunteers and placed into tubes containing an anticoagulant. The samples were centrifuged at room temperature at 3.000 rpm for 10 minutes to separate the erythrocytes, which were then washed with isosaline solution and resuspended to a 10% v/v concentration.

Subsequently, 0.5 mL of the RBC suspension was mixed with 1 mL of phosphate-buffered saline (PBS, pH 7.4), 2 mL of hypotonic saline solution, and 1 mL of the honey sample at concentrations of 6.25 to 100 ppm. For the positive control, 1 mL of sodium diclofenac at the same concentrations was used. The mixtures were incubated at 56°C for 30 minutes, followed by centrifugation at 5.000 rpm for 10 minutes. The absorbance of the supernatant was measured at 450 nm using a UV-VIS spectrophotometer. Sodium diclofenac was selected as the positive control because it is a well-established non-steroidal anti-inflammatory drug (NSAID) known to stabilize erythrocyte membranes, allowing a pharmacologically relevant comparison with the test compound (Hemalatha Nakka et al., 2025). The percentage of hemolysis and membrane stability were calculated using the following equations:

% Hemolysis = [((Sample absorbance)/(Negative control absorbance)) x 100] .....[1]

% Stability = 100 - [((Sample absorbance)/(Negative control absorbance)) x 100] .....[2]

2. Protein Denaturation Assay

The anti-inflammatory activity through protein denaturation inhibition was evaluated following the procedure described by (Yodha et al., 2024), with slight modifications. A Tris-buffered saline (TBS) solution was prepared by dissolving 870 mg of NaCl and 120 mg of Tris base in 100 mL of distilled water. Bovine serum albumin (BSA) solution was made by dissolving 200 mg of BSA in 100 mL of TBS, and the pH was adjusted to 6.2 – 6.5 using glacial acetic acid (Merck®, Germany).

The positive control solution was prepared by dissolving 50 mg of sodium diclofenac in 20 mL of ethanol, then diluting it to 100 mL to obtain a 500ppm stock solution, which was further diluted to concentrations of 6.25 to 100 ppm. The honey test solution was prepared by dissolving 1 gram of honey in 10 mL of 70% ethanol, then diluting it to 100 mL to obtain a 10.000 mg/L stock solution, which was also diluted to the same series of concentrations. The assay was conducted by mixing 2 mL of the test solution or positive control with 2 mL of 0.2% BSA solution, incubating for 30 minutes at 25°C, heating at 72°C for 5 minutes, and then cooling for 25 minutes. Absorbance was measured at 660 nm using a UV-Vis spectrophotometer (Techcomp®2501, Shanghai). The percentage inhibition of protein denaturation was calculated using the following formula:

$$\%\ \text{Inhibition}\ (\text{Anti-inflammatory Activity}) = \frac{\text{Absorbance of control-Absorbance of Sample}}{\text{Absorbance of control}} \times 100 \quad \text{.....[3]}$$

The percentage inhibition results for each concentration were plotted on a linear regression curve to determine the IC_{50} value (the concentration at which 50% inhibition occurs). Samples exhibiting more than 20% inhibition were considered to possess anti-inflammatory activity.

Radical Nitric Oxide (NO) Inhibition Assay

The nitric oxide inhibition assay was conducted based on the method described by (Suryani et al., 2023) with minor modifications. Sample solutions were prepared at concentrations ranging from 31.25 to 100 ppm, with Vitamin C at the same concentrations serving as the positive control. A total of 0.1 mL of each sample solution was mixed

with 0.1 mL of 10 mM sodium nitroprusside (SNP) prepared in 20 mM phosphate buffer (pH 7.4), and incubated for 2 hours at 37°C. During incubation, SNP reacts with oxygen to produce nitrite ions. Subsequently, 0.1 mL of Griess reagent was added to each mixture to form a pink-colored azo dye (chromophore). The absorbance was then measured at 540 nm using a microplate reader. The percentage of nitric oxide inhibition was calculated based on the reduction in absorbance of the SNP solution in the presence of the sample, using the following formula:

$$\text{Inhibition (Anti-inflammatory Activity)NO} = \frac{\text{Absorbance of control-Absorbance of Sample}}{\text{Absorbance of control}} \times 100 \quad \text{.....[4]}$$

Statistical Analysis

Statistical analysis was performed using SPSS version 29. An independent t-test was used to compare the anti-inflammatory activity between the treatment and control groups. If the assumption of normal distribution was not met, the analysis was continued with the non-parametric Mann-Whitney U test. A p-value of less than 0.05 was considered to indicate a significant difference between the two groups.

RESULTS AND DISCUSSION

Identification of Chemical Composition of Forest Honey using GC-MS

The GC-MS/MS analysis of forest honey identified several compounds present in honey, each characterized by specific retention times (RT), molecular weights, and chemical formulas, as presented in Table 1. These compounds exhibit diverse phytochemical profiles and are known to possess potential biological activities, including anti-inflammatory (Abusuliman et al., 2023; Amin et al., 2022; Aparna et al., 2012; Elwekeel et al., 2023; Samarghandian et al., 2017), anticancer (Nair et al., 2020), antioxidant (Amin et al., 2022; Elwekeel et al., 2023), antibacterial (Shaaban et al., 2021), and antimicrobial properties (Khromykh et al., 2022). The chromatogram depicted in Figure 1 illustrates the identified peaks of these volatile compounds, reflecting the chemical diversity in forest honey. The presence of these bioactive compounds supports the potential of forest honey as a natural therapeutic agent. Volatile constituents such as phenolics, terpenoids, and other aromatic compounds are key indicators of the biological properties of forest honey, contributing to its various pharmacological effects.

Table 1. Chemical Composition of Forest Honey Based on GC-MS Analysis

No.	Retention Time	Molecular Weight	Match	Reverse Match	Probability (%)	Formula	Compounds Name	Biological Activity	Compound Class
1	3.148	196	658	711	5.62	$C_{13}H_{12}N_2$	Propanedinitrile, (1-methylethenyl)(phenylmethyl)-	Antimicrobial	Nitrile / Volatile
2	4.212	184	606	619	3.77	$C_{11}H_{20}O_2$	2-Propenoic acid, 6-methylheptyl ester	Antioxidant	Ester / Volatile
3	5.583	154	610	701	10,1	$C_{10}H_{18}O$	1-Hexyne, 3-ethoxy-3,4-dimethyl-	Antimicrobial, Antibacterial	Alkyne / Volatile
4	7.22	138	718	731	14.3	$C_{10}H_{18}$	Cyclopentene, 1,2,3,4,5-pentamethyl-	Anti-imflammatory, antioxidant	Terpene / Volatile
5	8.280	138	683	695	5.03	$C_8H_{10}O_2$	Ethyl p-hydroxybenzoate	Antioxidant	Ester / Volatile
6	10.028	206	700	700	25.3	$C_{14}H_{22}O$	2,4-Di-tert-butylphenol	Anti-imflammatory, antioxidant, anticancer	Phenol / Semi-volatile
7	11.162	213	601	714	2.41	$C_{12}H_{23}NO_2$	1-Hexyl-1-nitrocyclohexane	Antimicrobial	Nitro compound / Semi-volatile
8	12.031	266	552	601	5.74	$C_{15}H_{22}O_4$	(2E,9Z)-5-Hydroxy-10-(hydroxymethyl)-3,7,7-trimethylcycloundeca-2,9-diene-1,6-dione	Anti-inflammatory, antioxidant	Diterpenoid / Non-volatile
9	13.640	380	572	572	3.09	$C_{25}H_{48}O_2$	Behenyl acrylate	Anti-inflammatory	Ester / Non-volatile
10	15.504	270	715	733	19.6	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester	Anti-inflammatory, antibacterial	Fatty acid ester / Non-volatile
11	16.035	256	621	626	8.45	$C_{16}H_{32}O_2$	n-Hexadecanoic acid	Anti-inflammatory, antioxidant	Fatty acid / Non-volatile
12	16.522	298	578	583	11.8	$C_{19}H_{38}O_2$	Ethyl 14-methyl-hexadecanoate	Antioxidant, antibacterial	Fatty acid ester / Non-volatile

Table 1. Continued

13	16.795	298	704	709	26.5	$C_{19}H_{38}O_2$	i-Propyl 14-methyl-pentadecanoate	Antioxidant, antijamur	Fatty acid ester / Non-volatile
14	19.127	568	578	578	6.41	$C_{35}H_{68}O_5$	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester	Anti-inflammatory, antibacterial	Triglyceride / Non-volatile
15	21.601	290	703	713	16.0	$C_{18}H_{26}O_3$	2-Ethylhexyl trans-4-methoxycinnamate	Anti-inflammatory, antibacterial	Ester / Non-volatile
16	22.699	258	639	645	14.1	$C_{14}H_{26}O_4$	Hexanedioic acid, mono(2-ethylhexyl)ester	Anti-inflammatory, antibacterial	Dicarboxylic acid ester / Non-volatile
17	24.455	414	692	698	1.89	$C_{27}H_{55}Cl$	Heptacosane, 1-chloro-	Antibacterial	Alkane / Non-volatile
18	24.88	410	691	712	1.84	$C_{28}H_{58}O$	Isobutyl tetracosyl ether	Antibacterial, antioxidant	Ether lipid / Non-volatile

The compounds identified in forest honey using GC-MS/MS were matched against the NIST library. The match scores ranged from 552 to 715, while the probability values ranged from 1.84% to 26.5% (Table 1). Only compounds with a match score above 550 and a probability above 1.8% were considered for further discussion. These values indicate a moderate to high confidence in the identification of the detected compounds.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the honey sample identified several compounds with notable anti-inflammatory potential. One prominent compound detected was 2,4-Di-tert-butylphenol (2,4-DTBP), recognized for its significant antioxidant properties that help mitigate oxidative stress, a key factor in inflammation (Aga et al., 2023). This suggests that 2,4-DTBP contributes to the honey's potential as a natural anti-inflammatory agent. Additionally, the analysis revealed the presence of (2E,9Z)-5-Hydroxy-10-(hydroxymethyl)-3,7,7-trimethylcycloundeca-2,9-diene-1,6-dioneand and Hexanedioic acid, mono(2-ethylhexyl) ester. These compounds are known to inhibit the NF-κB pathway, which regulates pro-inflammatory cytokines, thereby supporting the hypothesis that honey contains bioactive substances contributing to its therapeutic effects, particularly in reducing inflammation (Rossi & Marrazzo, 2021). These findings reinforce the potential use of honey as a natural agent in inflammation therapy, although further research is necessary to elucidate the specific mechanisms of each identified compound.

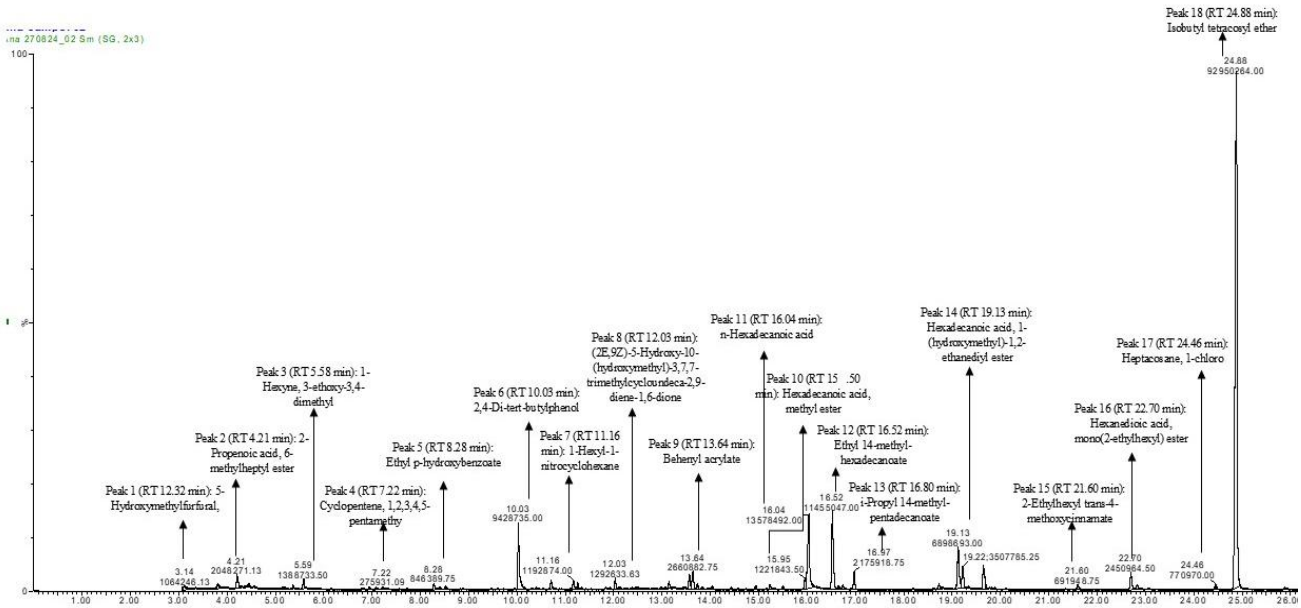


Figure 1. Gas chromatogram profile of forest honey obtained through gas chromatography/mass spectrometry (GC-MS)

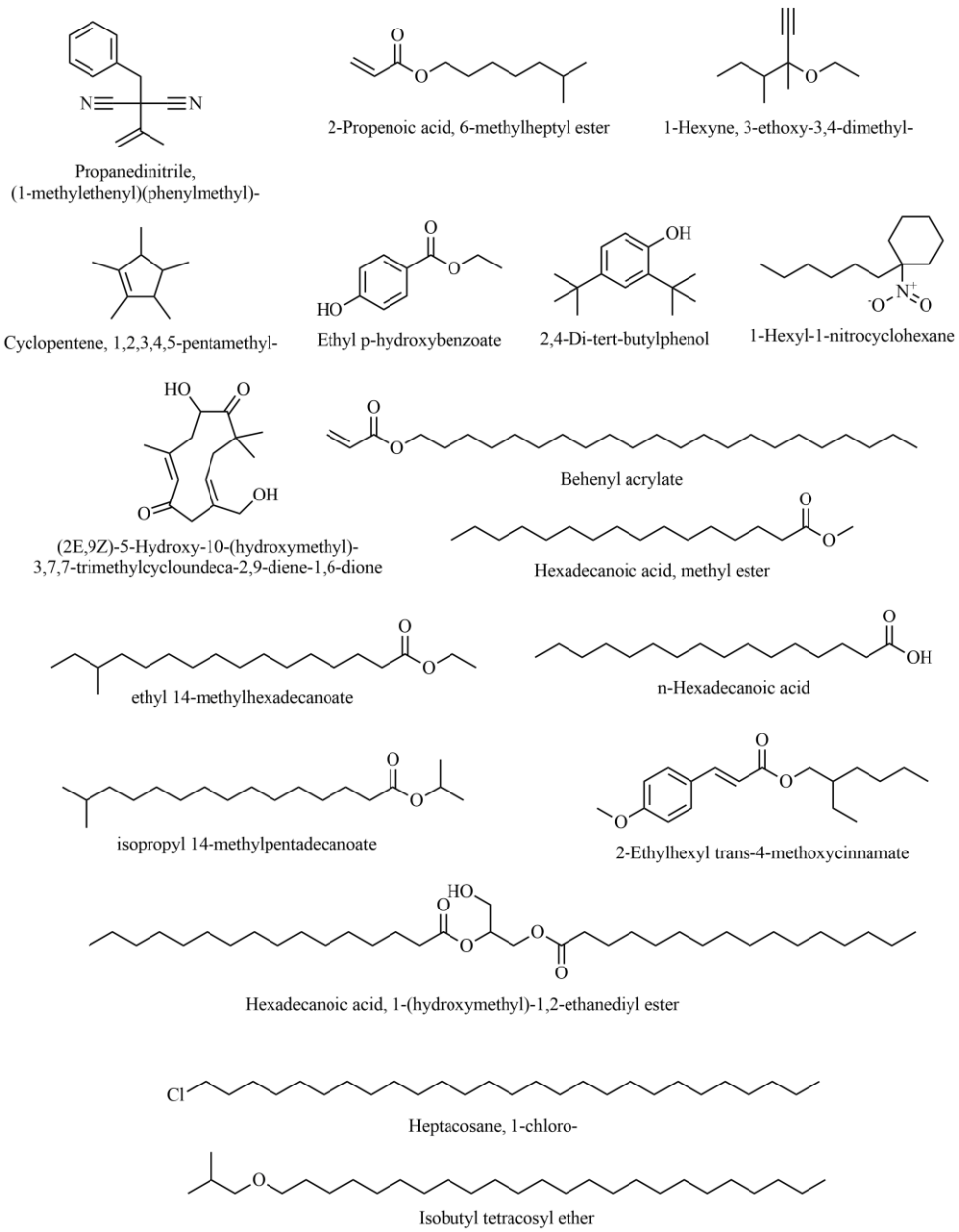


Figure 2. Structure of identified metabolites based on GC-MS analysis

Red Blood Cell Membrane Stabilization Test

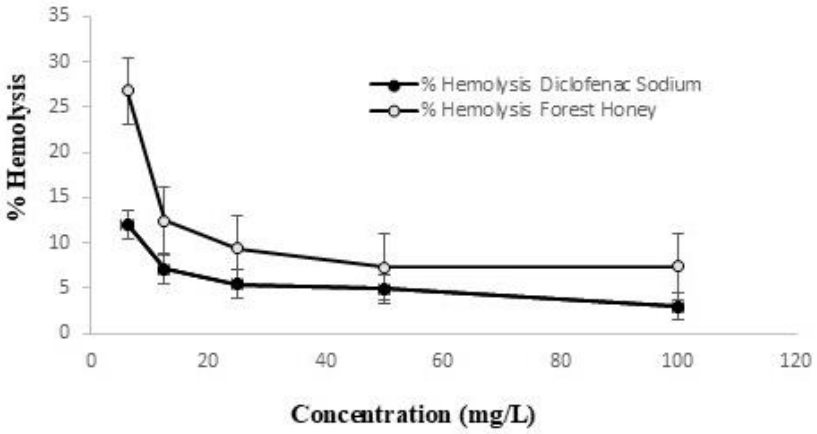
The Human Red Blood Cell (HRBC) membrane stabilization assay is commonly employed to evaluate in vitro anti-inflammatory activity, as the HRBC membrane closely resembles the lysosomal membrane. When these membranes are compromised, cyclooxygenase enzymes are released, converting arachidonic acid into prostaglandins, key mediators of inflammation (Karrat et al., 2022).

In this assay, the percentage of membrane stability is inversely related to hemolysis, higher membrane stability indicates lower hemolysis and, consequently, greater anti-inflammatory potential. Therefore, a compound's ability to prevent hemolysis and maintain cell membrane stability can indicate its potential anti-inflammatory effects. In this study, forest honey demonstrated a notable capacity to stabilize red blood cell membranes, although its efficacy was slightly lower than sodium diclofenac. At a concentration of 6.25 mg/L, forest honey achieved a membrane stability of 73.16% (hemolysis 26.84%), whereas sodium diclofenac reached 87.96% (hemolysis 12.04%). At the highest concentration tested (100 mg/L), forest honey exhibited a membrane stability of 92.55% (hemolysis 7.45%), compared to 97.00% (hemolysis 3.00%) for sodium diclofenac.

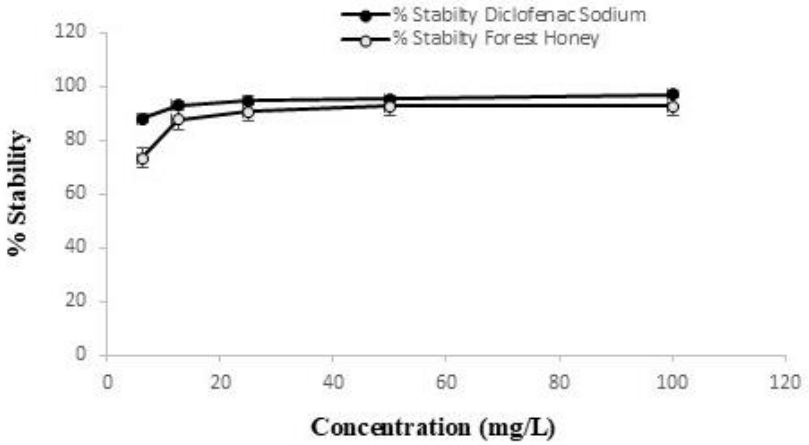
These findings, detailed in Table 2 and illustrated in Figure 3, suggest that forest honey possesses significant membrane-stabilizing activity, and the inverse relationship between hemolysis and membrane stability confirms its potential anti-inflammatory effects, albeit marginally less potent than sodium diclofenac.

Table 2. Percentage values of stability and hemolysis of ethanol extracts of East Kolaka Forest Honey and Sodium Diclofenac

No.	Concentration (mg/Liter)	% Hemolysis		% Stability	
		Diclofenac Sodium	East Kolaka Forest Honey	Diclofenac Sodium	East Kolaka Forest Honey
1	6.25	12.04	26.84	87.96	73.16
2	12.5	7.07	12.46	92.93	87.54
3	25	5.43	9.39	94.57	90.61
4	50	4.90	7.33	95.10	92.67
5	100	3.00	7.45	97.00	92.55



(a) Hemolysis activity of forest honey extract on human red blood cells (HRBC), with diclofenac sodium as a positive control. Data are presented as mean ± SD (n=3).



(b) Stability activity of forest honey extract on human red blood cells (HRBC), with diclofenac sodium as a positive control. Data are presented as mean ± SD (n=3).

Figure 3. In vitro anti-inflammatory activity test of East Kolaka Forest Honey

Protein Denaturation Test

Forest honey is a natural product rich in bioactive compounds, including flavonoids, phenolics, vitamins, and enzymes, contributing to its various pharmacological benefits, notably its anti-inflammatory effects (Hulea et al., 2022; Lawag et al., 2023). These properties can be evaluated through methods such as the Bovine Serum Albumin (BSA)

protein denaturation assay, which assesses protein stability under inflammatory conditions. Inflammation is the body's biological response to harmful stimuli like infections, injuries, or irritants (Fristiohady et al., 2019; Fristiohady A, 2020). In this assay, denaturation occurs through *thermal denaturation* of BSA, where heat exposure disrupts the protein's secondary and tertiary structures, exposing hydrophobic groups that aggregate and increase the turbidity of the solution. No color reagent is used in this method; instead, the measurement is based on *turbidity* changes, not color formation. The absorbance of the reaction mixture is measured at a wavelength of 660 nm, selected to detect turbidity caused by protein aggregation. Light at this wavelength is sensitive to particle scattering rather than pigment absorption, thus effectively representing the degree of protein denaturation (Yodha et al., 2024).

The control absorbance refers to the absorbance of the system without the addition of the honey sample, which represents maximum protein denaturation. A reduction in absorbance in the presence of honey indicates its protective effect against protein denaturation. This analytical principle is classified as a *turbidimetric assay*, distinct from colorimetric assays such as Lowry, which quantify total protein content based on chemical color reactions. (Alina et al., 2024; Galicia-Moreno et al., 2021). Research indicates that forest honey exhibits significant inhibition of protein denaturation, as evidenced by decreased absorbance values and increased inhibition percentages.

This suggests that the bioactive compounds in forest honey interact with proteins to maintain their structural stability, thereby preventing denaturation caused by inflammation. The IC_{50} value of forest honey was found to be 12.70 ± 0.02 mg/L, while that of sodium diclofenac was 5.83 ± 3.99 mg/L. A compound is considered to have powerful anti-inflammatory activity if the IC_{50} is less than 10 mg/L, strong at IC_{50} 10–30 mg/L, moderate at IC_{50} 31–50 mg/L, weak at IC_{50} 51–100 mg/L, and very weak at IC_{50} greater than 100 mg/L (Yodha et al., 2024).These findings underscore the potential of forest honey as a natural agent for managing inflammation.

Table 3. The IC_{50} value of honey based on anti-inflammatory test

No.	Sample	Mean±SD
1	East Kolaka Forest Honey	12.70 ± 0.02 mg/L
2	Diclofenac Sodium	5.83 ± 3.99 mg/L

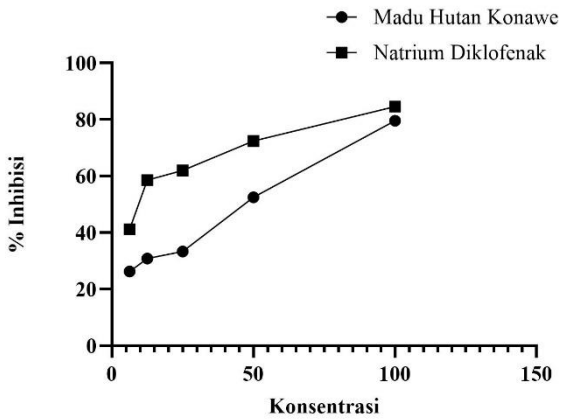


Figure 4. Anti-inflammatory of East Kolaka forest honey with sodium diclofenak as a control. Data are presented as mean±SD (n=3)

Radical Nitric Oxide (NO) Inhibition Assay

Nitric oxide (NO) is a free radical gas molecule that is water-soluble and can easily diffuse across cell lipoprotein membranes. While NO plays an essential role in various physiological processes, its excessive production during inflammation can be harmful. It reacts with oxygen (O_2) and hydrogen ions (H^+/H^-) to form highly reactive compounds such as peroxynitrite, which can damage tissues, accelerate cellular aging, and trigger both acute and chronic inflammatory responses (Suryani et al., 2023).

In this study, the ability of forest honey from East Kolaka to inhibit NO production was assessed using the sodium nitroprusside (SNP) method. The results revealed that East Kolaka forest honey exhibited an IC_{50} value of 2.97 ± 1.34 mg/L, while the

positive control, vitamin C, showed a lower IC_{50} of 1.07 ± 0.39 mg/L ($p < 0.05$). The IC_{50} value represents the concentration required to inhibit 50% of NO production, with lower values indicating stronger inhibitory activity. The complete data is presented in Table 4 and Figure 4. According to activity classification, East Kolaka forest honey falls within the category of very strong activity ($IC_{50} < 10$ mg/L), although still below the potency of pure antioxidant vitamin C.

This strong inhibitory effect can be linked to the bioactive compounds identified in the honey by GC-MS analysis. Compounds such as 2,4-Di-tert-butylphenol, hydroxylated diterpenoids, and various fatty acid esters are known for their antioxidant properties, enabling them to scavenge NO radicals. Additionally, some of these compounds may suppress inducible nitric oxide synthase (iNOS) or inhibit NF-κB signaling, further reducing NO production (Sukmawati et al., 2023)

Vitamin C was selected as a positive control because of its known ability to directly scavenge NO free radicals, whereas sodium diclofenac was not used as it acts via cyclooxygenase (COX) enzyme inhibition rather than direct NO suppression (Suryani et al., 2023). These findings confirm the promising potential of East Kolaka forest honey as a natural anti-inflammatory agent through its NO inhibitory mechanism, which could be relevant for managing both acute and chronic inflammation.

Table 4. The IC_{50} value of honey based on anti-inflammatory test

No.	Sample	Mean±SD
1	East Kolaka Forest Honey	2.97±1.34 mg/L
2	Vitamin C	1.07±0.39 mg/L

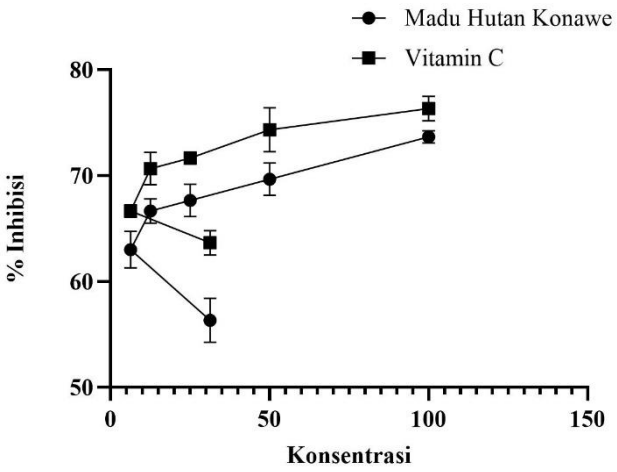


Figure 4. Anti-inflammatory of East Kolaka forest honey with vitamin c as a control. Data are presented as mean±SD (n=3)

Statistical analysis in this study was conducted to determine the differences in IC_{50} values of red blood cell (RBC) membrane stabilization, BSA protein denaturation inhibition, and nitric oxide (NO) production inhibition between East Kolaka forest honey and the positive control used. The statistical test was performed using an independent t-test, as only two groups of data were analyzed. The significance level was set at $p < 0.05$. The results showed statistically significant differences in all parameters, with the positive control exhibiting lower IC_{50} values compared to East Kolaka forest honey, indicating stronger biological activity.

CONCLUSION

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of East Kolaka forest honey identified several volatile bioactive compounds, including phenolics, terpenoids, and other aromatic substances known for their antioxidant, anti-inflammatory, antimicrobial, and therapeutic properties. The anti-inflammatory potential was evaluated through three in vitro methods: protein denaturation inhibition, red blood cell (RBC) membrane stabilization, and nitric oxide (NO) inhibition assays. in the RBC membrane stabilization assay, East Kolaka forest honey displayed increased

membrane stability and decreased hemolysis percentage with increasing concentrations, supporting its anti-inflammatory potential by protecting erythrocyte membranes against lysis.

In the protein denaturation assay, East Kolaka forest honey demonstrated an IC_{50} value of 12.70 ± 0.02 mg/L, while the positive control, diclofenac sodium, showed a lower IC_{50} of 5.83 ± 3.99 mg/L. These results suggest that although diclofenac sodium has stronger inhibitory activity, forest honey still exhibited moderate anti-inflammatory potential through protein stabilization. Additionally, In the NO inhibition assay, East Kolaka forest honey showed an IC_{50} value of 2.97 ± 1.34 mg/L, compared to 1.07 ± 0.39 mg/L for vitamin C, a standard antioxidant and anti-inflammatory agent.

This indicates that the honey sample possessed strong nitric oxide scavenging activity, approaching that of pure vitamin C. These findings confirm the promising anti-inflammatory and antioxidant activities of East Kolaka forest honey and highlight its potential as a natural therapeutic alternative, particularly in inhibiting nitric oxide production, preventing protein denaturation, and stabilizing cell membranes.

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